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Degradation of 2-chloroethanol by wild type and mutants of *Pseudomonas putida* US2

Uwe J. Strotmann[1], Marjan Pentenga[2], and Dick B. Janssen[2]

[1] Institute of Microbiology, University of Münster, Corrensstrasse 3, D-4400 Münster, Federal Republic of Germany
[2] Department of Biochemistry, Groningen Biotechnology Centre, University of Groningen, Nijenbrogh 16, 9747 AG Groningen, The Netherlands

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Abstract. A strain of *Pseudomonas putida* was isolated that was able to degrade 2-chloroethanol. The degradation proceeded via 2-chloroacetaldehyde and chloroacetate to glycolate. In crude extracts the enzymes for this degradation pathway could be detected. All enzymes proved to be inducible. The dehalogenase that catalyzed the dehalogenation of chloroacetate to glycolate was further characterized. It consisted of a single polypeptide chain with a molecular mass of 28 kDa. After induction the dehalogenase was expressed at a high level. In a mutant resistant to high concentrations of 2-chloroethanol the dehalogenase was no longer expressed. The mechanism of resistance seemed to be due to the inability to convert chloroacetate and export of this compound out of the cell.

Key words: Chloroethanol degradation – *Pseudomonas putida* – Degradative enzymes – Dehalogenase – Chloroethanol resistance

In chemical industry large amounts of short-chain chlorinated aliphatic organics are produced. These chemicals are used as organic solvents, degreasing agents, pesticides and intermediates for the synthesis of various other organic compounds. 2-Chloroethanol is such a widely used chemical. In vivo and in vitro it is metabolised by mammalian alcohol dehydrogenase to 2-chloroacetaldehyde, which is considered to be a mutagen (McCann et al. 1975). The degradation of 2-chloroethanol by bacteria also proceeds via the potentially toxic intermediates 2-chloroacetaldehyde and chloroacetic acid to glycolate (Stucki and Leisinger 1983; Janssen et al. 1987a). In *Xanthobacter autotrophicus* GJ10 the oxidation of 2-chloroethanol to 2-chloroacetaldehyde is catalyzed by a quinoprotein alcohol dehydrogenase and the subsequent conversion of 2-chloroacetaldehyde to chloroacetic acid by an NAD-dependent aldehyde dehydrogenase (Janssen et al. 1985). A dehalogenase is involved in dehalogenation of chloroacetic acid to glycolate.

We recently described a method for the rapid isolation of organisms capable of degrading chlorinated hydrocarbons (Strotmann and Röschenthaler 1987). One of the organisms that was isolated by this method is capable of degrading 2-chloroethanol. The physiology of this organism is presented in this report. Our special interests concern the degradative enzymes, their regulation and inhibition by metabolites. Since 2-chloroethanol is a toxic chemical that can inhibit growth of bacteria, we isolated resistant mutants of the degradative strain and studied the mechanism of resistance.

Offprint requests to: U. J. Strotmann, BASF AG, Emissionsüberwachung und Ökologie, D-6700 Ludwigshafen, Federal Republic of Germany

Non-standard abbreviations: CEO 2-chloroethanol; DCPIP 2,6-dichlorophenolindophenol; FPLC fast protein liquid chromatography; PAGE polyacrylamide gelelectrophoresis; PES phenazine ethosulfate; PMS phenazine methosulfate; PQQ pyrroloquinoline quinone

Materials and methods

Enrichment of a degradative strain and growth conditions

The enrichment of a 2-chloroethanol-degrading strain was carried out at 30°C with MMZ-medium (Janssen et al. 1984) containing 5 mM of 2-chloroethanol. As an inoculum water samples from the Rhine at Duisburg (FRG) were used. After repeated subculturing a pure isolate was obtained by streaking on indicator plate medium (Strotmann and Röschenthaler 1987) containing 20 mM of 2-chloroethanol. By means of a Oxiferm II-system (Hoffmann-La-Roche, Basel) the pure isolate could be identified as a strain of *Pseudomonas putida* and designated US2. For further experiments, strain US2 was grown in glass bottles filled to maximally one-fifth of their volume with MMZ medium supplemented with 0.01% yeast extract. Cultivation was carried out at 30°C on a rotary shaker and cells were harvested in the late exponential growth phase.

Preparation of crude extract and enzyme assays

All operations were performed at 4°C. Cultures were harvested by centrifugation (10000 × *g*, 10 min, Sorvall Superspeed RC-5B-

centrifuge), washed twice with Tris-buffer (20 mM Tris-sulfate, 2 mM EDTA, pH 9.0) and resuspended in the same buffer (10 ml of buffer per 1 g fresh weight). After addition of 2 mM of β-mercaptoethanol the cells were disrupted by sonication. A crude extract was obtained by centrifugation (30000 × *g*, 30 min, 4° C).

Dehalogenase activities were measured by determining the production of chloride from chlorinated organic substrates. Chloride assays were carried out with a colorimetric assay (Bergmann and Sanik 1957). The formation of glycolate from chloroacetate was followed by the method of Dagley and Rodgers (1953). Dehalogenase activities were measured at 30° C in an incubation buffer (20 mM Tris-sulfate pH 9.0) including 20 mM of chlorinated substrate and 100 µl of enzyme (1 – 3 mg protein/ml of enzyme solution). To measure dehalogenase activity with low concentrations of chlorinated substrates, the decrease of pH due to liberation of protons in a low-buffered incubation mixture was recorded. This test was performed at 30° C in an incubation mixture containing, in a final volume of 5 ml: 2 mM Tris-sulfate buffer pH 8.0, 0.1 mM – 5 mM of chlorinated substrate, 50 µl of enzyme (1 – 3 mg protein/ml of enzyme solution). Determination of glycolate, produced from chloroacetate by the dehalogenase, was carried out by the colorimetric assay of Dagley and Rogers (1953).

2-Chloroethanol dehydrogenase was measured by following the 2-chloroethanol-dependent reduction of 2,6-dichlorophenolindophenol (DCPIP) spectrophotometrically at 600 nm in a coupled assay with phenazine ethosulfate (PES) as artificial electron acceptor (Janssen et al. 1984). The incubation mixture contained in a final volume of 1 ml: 38 mM Tris-sulfate pH 8.5, 15 mM NH_4Cl, 220 µM PES, 10 mM KCN, 100 µM DCPIP, 25 mM of substrate and 50 µl of enzyme solution (1 – 3 mg protein/ml of enzyme solution). The reaction was started by the addition of substrate (2-chloroethanol) after equilibration of the other components and was carried out at 30° C.

Chloroacetaldehyde dehydrogenase and NAD-dependent alcohol-dehydrogenase activities were assayed spectrophotometrically at 30° C by following the reduction of NAD at 340 nm. Reaction mixtures contained in a final volume of 1 ml: 100 mM Tris-sulfate pH 9.0, 5 mM NAD, 10 mM of substrate and 30 µl of enzyme solution (1 – 3 mg of protein/ml enzyme solution). The reaction was started by the addition of substrate and was carried out at 30° C.

One unit of enzyme activity is defined as the activity catalyzing the formation of 1 µmol of the reaction product per minute. Protein concentrations were determined by the method of Bradford (1976).

Purification and characterization of chloroacetate dehalogenase

Chloroacetate dehalogenase was partially purified from 2-chloroethanol-grown cells by preparative native polyacrylamide gelelectrophoresis. Dehalogenase activities could be visualized on polyacrylamide gels by $AgNO_3$-precipitation of chloride liberated upon incubation of the gels with chlorinated substrate (Hardman and Slater 1981). From comparable gels, which were not treated with $AgNO_3$-solution, the dehalogenase band was cut out and the dehalogenase was eluted from the gel by electroelution (Biotrap-system by Schleicher and Schüll). All procedures were performed at 4° C. The buffer used for eletrophoresis and electroelution contained 25 mM Tris, 192 mM glycine, 1 mM EDTA and 2 mM β-mercaptoethanol. An amount 2.1 mg of protein was applied to this procedure and 0.13 mg of protein was recovered after electroelution. The eluted protein was checked for enzymatic activity by a dehalogenase assay and for purity by SDS-PAGE on 10 or 15% polyacrylamide according to the method of Laemmli (1970). Molecular mass markers were ovotransferrin (78 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (17 kDa) and cytochrome *c* (12.3 kDa). Scanning of SDS-gels was performed with a Hirschmann gel scanner.

FPLC-gel filtration (Pharmacia system) with a Superose-12-column was used for determining the native molecular weight of the dehalogenase. For equilibration of the column and elution of the enzyme, a potassium-phosphate-buffer (100 mM, pH 7.4, 2 mM β-mercaptoethanol and 1 mM EDTA) was used. The eluted fractions were checked for dehalogenase activity by measuring the liberation of chloride upon incubation with chloroacetate. The following proteins were used for calibration of the column: aldolase (158 kDa), bovine serum albumine (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (26 kDa).

Isolation of mutants resistant to 2-chloroethanol

P. putida US2 was grown in MMZ-medium with 20 mM citrate as sole carbon source. In the late exponential phase, the culture was diluted with sterile NaCl-solution (0.9%; dilution steps: 10^{-1} to 10^{-5}). An aliquot of 100 µl of each dilution step was spread on a minimal medium plate containing 5 mM citrate and 200 mM of 2-chloroethanol. The inoculated plates were incubated at 30° C for 5 days. Fourty colonies were picked and streaked again on the same medium to verify resistance. From these strains stock cultures were prepared. The mutants were desiganted as US2M1 – US2M40.

Gas chromatography

2-Chloroethanol was quantitatively determined by means of a Shimadzu GC9A gaschromatograph equipped with a 1.80 m Porapak Q column (80 – 100 mesh). Further details were previously described (Strotmann and Röschenthaler 1987). The determination of chloroacetate after methylation by GC-MS was performed according to the method of Stucki and Leisinger (1983) with a capillary gaschromatograph (gas chromatograph Shimadzu GC8, mass spectrometer Varian CH7A) equipped with a fused silica capillary column (50 m) coated with SE 54 cross-linked film as stationary phase. Complete mass spectra in the appropriate range were recorded. Identification was done by comparing these spectra with reference spectra. Further conditions were previously described (Strotmann 1988).

Results

Properties of Pseudomonas putida US2

P. putida US2 is a strain that was isolated from the river Rhine. With help of the indicator plate method the isolation of a pure culture could be performed in two weeks. The initial characterization of the strain revealed that it was a Gram-negative, oxidase-positive, catalase-positive mobile rod (length: 2 – 3 µm). The strain was able to grow on glucose, sucrose, glycerate, glycolate, citrate, succinate, ethanol, ethylene glycol and acetate. Ammonium and nitrate were used as nitrogen sources. On *Pseudomonas* F-agar (King et al. 1954) the strain formed yellow-green fluorescent pigments. Apart from nonhalogenated compounds, 2-chloroethanol, chloroacetate and 2-chloropropionate could support growth. Dichloroacetate and glyoxylate supported weak growth. Methanol, trichloroacetate, 2,2-dichloropropionate and 1,2-dichloroethane were not utilized as carbon sources.

Degradation of 2-chloroethanol

2-Chloroethanol supported exponential growth of strain US2 with a growth rate constant of 0.11 h^{-1} (Fig. 1).

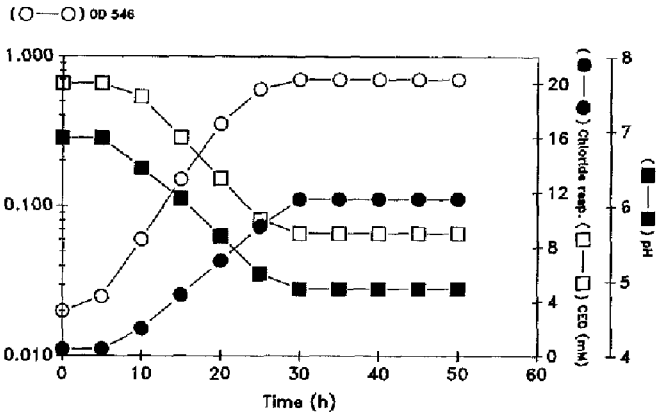

Fig. 1. Growth of *Pseudomonas putida* US2 in MMZ-medium with 20 mM of 2-chloroethanol (CEO). Growth (OD_{546}), pH, chloride and chloroethanol concentration were followed

Growth was accompanied by chloride and proton release, which strongly exceeded uninoculated controls. In shake flash cultures, hydrochloric acid production caused a drop in pH and no further degradation occurred when the pH was below 5 (Fig. 1). When yeast extract was added, the growth rate and the degradation rate were enhanced. The optimization of the yeast extract-concentration in the medium yielded an optimal concentration of 0.02% – 0.03% (w/v), which gave a growth rate of 0.35 h^{-1}.

Although being used as a growth substrate, 2-chloroethanol could inhibit growth of *P. putida* US2 in liquid medium. When growing on an easily utilizable carbon source such as glucose, high concentrations of 2-chloroethanol (> 30 mM) inhibited growth. The inhibition was dependent on the concentration of 2-chloroethanol. Addition of 2-chloroethanol at concentrations greater than 70 mM caused lysis of the cultures. After further incubation of the lysed cultures some resistant bacteria started to grow which proved to be insensitive even to 200 mM of 2-chloroethanol. When strain US2 was incubated in minimal medium with low concentrations of 2-chloroethanol (up to 20 mM) the cells formed visible clusters before starting growth. This formation of clusters was also a sign for the toxic effects of 2-chloroethanol even at lower concentrations. Clusters were not formed when strain US2 was incubated with chloroacetate, glucose or citrate.

Degradative enzymes, their regulation and inhibition by metabolites

To investigate the route of 2-chloroethanol metabolism, crude extracts were used and tested for specific enzymatic activities as described by Stucki and Leisinger (1983) and Janssen et al. (1987a). Crude extracts contained an inducible PES-dependent chloroethanol dehydrogenase and an NAD-dependent chloroacetaldehyde dehydrogenase activity. An inducible chloroacetate dehalogenase activity was also detected (Table 1). 2-Chloroethanol was the best inducer for all enzymes. Ethanol also was an inducer of the PES-dependent alcohol dehydrogenase and of the NAD-dependent aldehyde dehydrogenase, but was less efficient in induction of the dehalogenase. Chloroacetate was an effective inducer of the dehalogenase but a weaker inducer of PES-dependent alcohol dehydrogenase and NAD-dependent aldehyde dehydrogenase.

Further properties of the investigated enzymes are summarized in Table 2. Ammonia had an activating effect on the activity of the PES-dependent alcohol dehydrogenase. The enzyme activity was enhanced by as much as 38% when ammonia was present in the incubation mixture compared to a control without ammonia. PES seemed to be a better electron acceptor for this enzyme than phenazine methosulfate (PMS). When PES was replaced by PMS the activity decreased by 42%. PES-dependent alcohol dehydrogenase activity was highest with secondary alcohols (2-propanol, 2-pentanol, 2-hexanol) and long chain primary alcohols (nonanol, undecanol) with activities in the range of 2.5 to 2.9 U/mg protein. The activity towards halogenated alcohols, as 2-chloroethanol and 2-bromoethanol and towards short-chain primary alcohols (methanol, ethanol, butanol, heptanol) was lower (0.9 to 1.3 U/mg protein). Also diols (ethylene glycol, 1,5-pentanediol, 1,6-hexanediol) were oxidized by the enzyme with rates between 1.0 and 1.6 U/mg protein.

Besides PES-dependent alcohol dehydrogenase activity, crude extracts of strain US2 also contained an alcohol dehydrogenase that was active with NAD as a cofactor. Only very low ethanol dehydrogenase activity could be detected with NADP as a cofactor with specific activity less than 15 mU/mg protein in crude extracts from ethanol grown cells. The activity of the NAD-dependent dehydrogenase was highest with primary alcohols with two to eight carbon atoms (140 to 170 mU/mg protein), but activities lower than 20 mU/mg protein were detected with diols. No activity was observed with halogenated alcohols. The enzyme was not produced constitutively, but was inducible with ethanol, 2-chloroethanol, chloroacetate and glucose (data not shown). The K_m value for ethanol was 0.38 mM and the optimal pH-range was between pH 8.0 and 9.0. Although serving as an inducer, 2-chloroethanol could inhibit the activity of this dehydrogenase. It was found that the inhibition was of a competitive type and that the inhibition constant K_i was 0.8 mM.

The oxidation of 2-chloroacetaldehyde to chloroacetate was catalyzed by an NAD-dependent aldehyde dehydrogenase at a rate of 72 mU/mg protein (Table 2). Substrates converted included acetaldehyde (153 mU/mg protein), propionaldehyde (84 mU/mg protein), butyraldehyde (48 mU/mg protein), hexanal (72 mU/mg protein) and octanal (36 mU/mg protein). The NAD-dependent aldehyde dehydrogenase also proved to be inducible; the best inducers were 2-chloroethanol and ethanol (Table 1). The K_m value of the enzyme for 2-chloroacetaldehyde was 27 μM. When testing the enzyme activity at higher concentrations of 2-chloroacetaldehyde a strong

Table 1. Regulation of PES-dependent chloroethanol dehydrogenase, NAD-dependent chloroacetaldehyde dehydrogenase and chloroacetate dehalogenase formation in *Pseudomonas putida* US2

Growth substrate	Specific activities in crude extracts [mU/mg protein]		
	PES-dependent chloroethanol dehydrogenase	NAD-dependent chloroacetaldehyde dehydrogenase	Chloroacetate dehalogenase
2-Chloroethanol	980	70	25,000
Chloroacetate	285	12	20,750
Ethanol	980	60	8,500
Succinate	430	1	2,000
Glucose	380	22	6,750
Citrate	205	1	7,000

Table 2. Some properties of the degradative enzymes of P. putida US2

Enzyme	Optimal range of pH	Spec. activity in crude extracts of induced cells [mU/mg protein]	Optimal range of temperature [°C]
PES-dependent chloroethanol dehydrogenase	9,0	981	40–45
NAD-dependent chloroacetaldehyde dehydrogenase	7.5–7.7	72	45
Chloroacetate dehalogenase	10.0	25,000	50

inhibition was observed. In presence of 10 mM 2-chloroacetaldehyde only 50% of the normal enzyme activity could be measured.

Characterization of chloroacetate dehalogenase

The dehalogenase present in crude extracts of strain US2 showed activity towards several chlorinated acids. The rates of conversion of dichloroacetate, 2-chloropropionate and 2.2-dichloropropionate were, respectively, 16%, 27% and 16% of the rate found with chloroacetate. Trichloroacetate and 2-chlorobutyrate were not hydrolyzed. Incubation of 20 mM chloroacetate for 3 min with 10.5 U of dehalogenase yielded 7 mM of chloride and 6.5 mM of glycolate, showing that the product of chloroacetate dehalogenation was glycolate. The K_m value for chloroacetate was determined to be 0.62 mM and the enzyme was irreversibly inhibited by sulfhydryl agents such as $HgCl_2$ (50 µM) and p-chloromercuribenzoate (50 µM). In crude extracts of induced cells, dehalogenase activities of about 25 U/mg protein could be measured (Table 1). Even in non-induced cells high levels of dehalogenase were present (2 U/mg of protein in succinate-grown cells).

A crude extract (2 mg protein) from 2-chloroethanol grown cells was subjected to polyacrylamide gelelectrophoresis under non-denaturating conditions (Fig. 2A). The dehalogenase band was cut out and the enzyme was obtained by electroelution. It was found that dehalogenase was still active after this one-step purification procedure. By SDS-PAGE, the eluted protein solution was found to be essentially free of contaminating proteins and the molecular mass of dehalogenase was determined to be 28 kDa (Fig. 2B). By FPLC chromatography on a superose-12-column the native molecular mass of dehalogenase was determined to be 30 kDa.

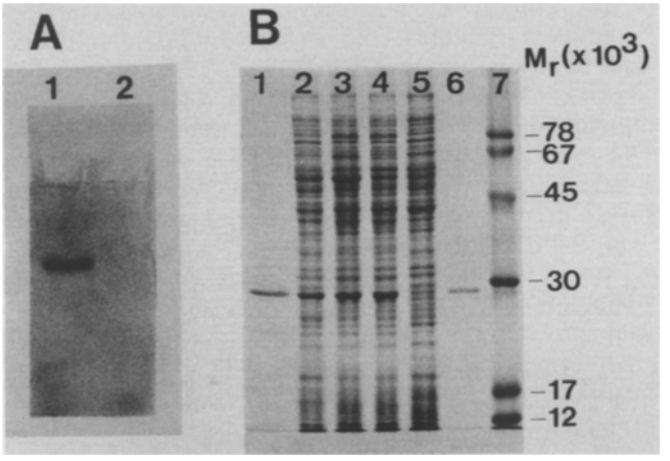

Fig. 2. **A** Native polyacrylamide gelelectrophoresis to visualize dehalogenase activity. Crude extracts were subjected to electrophoresis and dehalogenase was visualized with an activity stain. Extracts were prepared from strain *P. putida* US2 grown on 2-chloroethanol (1) and Nutrient Broth (2). **B** SDS-polyacrylamide gelelectrophoresis of crude extracts and purified dehalogenase. *Slots* are: (1), (6), purified dehalogenase, (2), (3), (4), crude extract of strain US2 grown on citrate, 2-chloroethanol and chloroacetate, respectively; (5), crude extract from mutant US2M1 grown on citrate; (7), marker proteins

Table 3. Enzymatic activities in crude extracts of wild-type cells and cells of mutant US2M1 grown in minimal medium with 20 mM ethanol and 20 mM 2-chloroethanol

Enzyme	Specific enzymatic activity [mU/mg protein]	
	Wild type	Mutant US2M1
PES-dependent chloroethanol dehydrogenase	950	750
NAD-dependent chloroacetaldehyde dehydrogenase	65	60
Chloroacetate dehalogenase	23,000	< 5

Therefore, dehalogenase of strain US2 seemed to consist of a single polypeptide chain with a molecular mass of about 28 kDa. SDS-PAGE of purified dehalogenase and crude extracts indicated that the dehalogenase was a predominant protein in crude extracts prepared from cells grown on citrate, chloroacetate or 2-chloroethanol (Fig. 2B). Scanning of the gel showed that about 10% of soluble protein in induced cells represented dehalogenase.

Physiology of resistance to 2-chloroethanol

Mutants of *P. putida* US2 resistant to 2-chloroethanol-concentrations up to 200 mM were easily selected on plates containing 5 mM citrate and 200 mM 2-chloroethanol. By this method 40 mutants could be isolated. One representative mutant, US2M1, was chosen for further studies. The mutant was no longer able to grow on chloroacetate or 2-chloroethanol as sole carbon source, but still utilized glycolate.

For testing what enzyme of the 2-chloroethanol assimilation pathway was lacking, cells of mutant US2M1 were grown in a medium containing ethanol and 2-chloroethanol as carbon sources. This medium was appropriate for induction of the degradative enzymes (Table 3). When activities were measured in crude extracts, it was found that only dehalogenase was lacking (Table 3). SDS-PAGE of crude extracts prepared from strain US2M1 grown on citrate indicated that the major protein band, representing dehalogenase in extracts from wild-type cells, was absent in the mutant (Fig. 2B). Furthermore, it was found that chloroacetate was excreted by mutant US2M1, when the supernatant of the grown culture was examined by GC-MS measurements as described by Stucki and Leisinger (1983). After 75 h of incubation of mutant US2M1 with 20 mM 2-chloroethanol and 20 mM ethanol a concentration of 12 mM chloroacetate was detected in the culture supernatant, while the chloride level was below 0.5 mM. Apparently, in mutant US2M1 resistance to high concentrations of 2-chloroethanol was accompanied by oxidation of the compound to chloroacetate which accumulated in the growth medium due to a lack of dehalogenase activity.

Discussion

In this report, we describe the isolation of a strain of *Pseudomonas putida* that is able to use 2-chloroethanol as growth substrate. The degradation of 2-chloroethanol was found to proceed via 2-chloroacetaldehyde to chloroacetic acid, which is hydrolytically dehalogenated to glycolate. The same route has been found in a 2-chloroethanol utilizing *Pseudomonas* strain (Stucki and Leisinger 1983) and a 1,2-dichloroethane degrading strain of *Xanthobacter autotrophicus* (Janssen et al. 1985).

In strain US2, three different dehydrogenases (two different alcohol dehydrogenases and one aldehyde dehydrogenase) were found. The expression of these alcohol dehydrogenases enabled the strain to metabolise a wide range of different alcohols, indicating that the enzymes are probably not specifically involved in chloroethanol metabolism. Similar alcohol dehydrogenase systems have been described in the literature previously (Barrett et al. 1980; Wyatt et al. 1987). The PES-dependent alcohol dehydrogenase of strain US2 was inducible and ammonia was needed for optimal activity. The same has been found with methylotrophic bacteria (Anthony 1982; Duine and Frank 1981a), which produce dehydrogenases that contain pyrroloquinoline quinone (PQQ) as a prosthetic group and are especially active with primary alcohols. Activity with halogenated alcohols was also reported. Furthermore, the substrate range in some organisms is not restricted to primary alcohols, but can also include secondary alcohols (Sahm et al. 1976; Yamanaka and Minoshima 1984; Schär et al. 1985; Duine and Frank 1981b). The PES-dependent enzyme of strain US2 was preferentially active with secondary alcohols, whereas a PQQ-containing dehydrogenase of *X. autotrophicus* GJ10 preferred primary alcohols as a substrate (Janssen et al. 1987a).

With help of the dehalogenase, chloroacetate is dehalogenated to glycolate. After induction a very strong expression of dehalogenase was observed, which caused the dehalogenase to be present as a predominant band of electropherograms of crude extracts. Dehalogenase activities up to 25 U/mg of protein were found in crude extracts of induced cells. This is about 50 times higher than the levels of haloacid-dehalogenase of other chloroethanol-utilizing bacteria (Stucki and Leisinger 1983; Janssen et al. 1985), 60 times higher than the activities usually found in haloacetate utilizing organisms (Hardman and Slater 1981; Motosugi and Soda 1983), and also 20 times higher than the dehalogenase activities of mutants that produced elevated dehalogenase activities after selection in continuous culture (Weightman and Slater 1980). Even in crude extracts of non-induced cells high activities of dehalogenase could be measured. Most probably, the expression of dehalogenase of strain US2 is due to a very efficient promoter. Therefore, it seems to be very interesting to analyze this strong expression on the genetic level.

The properties of the dehalogenase resemble in some points the characteristics of other dehalogenases that have been described before (Motosugi et al. 1982; Motosugi and Soda 1983). Systematically, this dehalo-

genase belongs to the class of the 2-haloacid dehalogenases (E.C. 3.8.1.2).

Intermediates formed during the degradation of 2-chloroethanol were chloroacetaldehyde and chloroacetate. Halogenated alkanoic acids like chloroacetate are known to cause different inhibitory effects (Weightman et al. 1985). Chloroacetaldehyde could inhibit NAD-dependent aldehyde dehydrogenase or chemically react with cell components if it accumulates. It also proved to be genotoxic agent in the SOS-chromotest (Strotmann 1988), in accordance with studies published earlier (Malaveille et al. 1975; Rannug et al. 1976; McCann et al. 1975). Toxicity of high concentrations of 2-chloroethanol was thus expected to be caused by intracellular accumulation of chloroacetaldehyde or chloroacetic acid. Several observations suggest that aldehydes are critical intermediates in the metabolism of alcohols. Thus, 2-chloroethanol was very toxic for a mutant of *X. autotrophicus* GJ10 that lacked chloroacetaldehyde dehydrogenase activity (Janssen et al. 1987a). Furthermore, mutants of *Acinetobacter* HO1-N that are resistant to allyl alcohol (Singer and Finnerty 1985) and mutants of a 1,2-dibromoethane hydrolyzing organism that are resistant to 2-bromoethanol (Janssen et al. 1987b) were found to be defective in the metabolism of alcohols, making these compounds essentially inert.

In *P. putida* US2M1, however, resistance to 2-chloroethanol must have a different cause since the activities of the enzymes converting 2-chloroethanol to chloroacetate were essentially unaffected in the mutant. The increased tolerance was accompanied by a loss of chloroacetate dehalogenase activity and accumulation of chloroacetate in the growth medium. Transformation of a xenobiotic compound to a metabolite that is subsequently excreted is not uncommon (Alexander 1985), although it is not clear why this would cause resistance here. Weightman et al. (1985) have also found an inverse correlation between dehalogenase expression and resistance to halogenated aliphatics. It was suggested that the mutation(s) involved had an effect both on dehalogenase activity and the expression of a permease, the latter causing resistance (Slater et al. 1985). If such a mechanism would cause increased tolerance to 2-chloroethanol in mutant US2M1, then either the expression of an exporting permease should be elevated, or the expression of a permease involved in (re)uptake of excreted chloroacetate should be reduced, as compared to the wild type strain. Genetic analysis of haloacetate dehalogenase genes, which is now under way for the *X. autotrophicus* GJ10 system (Janssen et al. 1989), is expected to give more insight into the regulation of transport and hydrolysis of 2-haloalkanoates and the correlation between resistance and enzyme levels.

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